

**Prediction models for assessing anthocyanins in grape berries by
fluorescence sensors: dependence on cultivar, site and growing
season**

**(Running title: Fluorescence-based prediction of grape
anthocyanins)**

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Abstract

Fluorescence sensors are useful tools for the non-destructive assessment of grape berry anthocyanins. Here, the anthocyanin indices ($ANTH_R$ and $ANTH_{RG}$) provided by the Multiplex (Mx) sensor were calibrated against wet chemistry. The dependence of anthocyanin prediction models on cultivar, season and site was studied on four cultivars in two Italian regions during three consecutive years. Prediction models in 2010 were built for the individual cultivars, one of which on two sites, all cultivars on one site, and all cultivars on both sites, and then validated on 2011–2012 data. The 2010 global model (all cultivars at both sites) gave relative prediction errors on anthocyanin content less than 14.1% ($ANTH_R$) and 21.3% ($ANTH_{RG}$). The $ANTH_{RG}$ was independent of season, maintaining a relative error of about 20% in both 2011 and 2012. In field applications of the calibrated Mx, it showed its ability to detect inter-plot and inter-season differences on both growing sites.

Fluorescence sensors are useful tools for the non-destructive assessment of grape berry anthocyanins. The Multiplex (Mx) sensor here studied provides two anthocyanin indices: $ANTH_R = \log(1/\text{Chl-fluorescence}_R)$ and $ANTH_{RG} = \log(\text{Chl-fluorescence}_R/\text{Chl-fluorescence}_G)$, based on the chlorophyll (Chl) fluorescence excited with red (R) and green (G) light. These indices were calibrated against wet chemistry. The dependence of anthocyanin prediction models on cultivar, season and site was studied on four cultivars in two Italian regions during three consecutive years. The 2010 global model (all cultivars at both growing sites) gave relative prediction errors on anthocyanin content less than 14.1% ($ANTH_R$) and 21.3% ($ANTH_{RG}$). The $ANTH_{RG}$ was independent of season, maintaining a relative error of about 20% in both 2011 and 2012. In field applications of the calibrated Mx, it showed its ability to detect inter-plot and inter-season differences on both growing sites.

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46 **Keywords**

47 Anthocyanins, fluorescence, in-field applications, non-destructive measurements, optical
48 sensors.

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51 **1. Introduction**

52 It is now well accepted that a premium wine trait depends on the quality of the grapes used to
53 produce it, and an important parameter that lends credibility to this premise is the colour of the
54 wine. Anthocyanins (Anths), the red pigments of the berry skins that define the colour of wines,
55 are good indicators of the so-called phenolic maturity of grapes. Phenolic maturity can be
56 assessed by measuring either total phenolics or skin anthocyanin content, which is closely
57 correlated with total phenolics (Kennedy, Matthews, & Waterhouse, 2002). In most red grape
58 varieties, Anths are located in the berry skin and accumulate, starting from véraison, until the
59 grapes ripen fully (Boss, Davies, & Robinson, 1996). In addition to technological maturity, i.
60 e. the acidity and sugar content of berries, phenolic maturity has now become the main concern
61 of viticulturists and oenologists in planning harvest time and in choosing the most appropriate
62 oenological techniques. Therefore, an accurate determination of the Anth content in the berries

is fundamental. This is routinely performed by using destructive ‘wet chemistry’ procedures, which are costly and time-consuming. Conversely, ~~Because of the large biological variability of grape-berry Anths, the introduction of optical sensors can be extremely advantageous – thanks to their non-destructive evaluation – as compared with destructive methods, which are costly and time-consuming.~~ The non-destructive evaluation of the by optical sensors can be extremely advantageous because of the large biological variability of grape-berry Anths. Conversely, destructive methods are costly and time-consuming.

~~Because of the large biological variability of grape-berry Anths, the introduction of optical sensors can be extremely advantageous – thanks to their non-destructive evaluation – as compared with destructive methods, which are costly and time-consuming.~~ In fact, a large number of samplings representative of the whole crop can be measured directly in the vineyard within relatively short time periods. Furthermore, the maturity process can also be followed on the same bunches during the entire season, by repeating the optical measurements at a high frequency. For this purpose, the Multiplex[®] sensor (FORCE-A, Orsay, France), a no-contact, hand-held optical device, has been developed (Cerovic, Moise, Agati, Latouche, Ben Ghazlen, & Meyer, 2008). It measures Anths in an indirect way that is based on the interference effect exerted by anthocyanin absorbance over chlorophyll fluorescence excitation in the green and red spectral regions (Agati, Meyer, Matteini, & Cerovic, 2007). The sensor provides two Anth indices: the ANTH_R based on a single signal, the far-red chlorophyll fluorescence under red excitation, also called the FERARI (Fluorescence Excitation Ratio Anthocyanin Relative Index) index (Ben Ghazlen, Cerovic, Germain, Toutain, & Latouche, 2010), and the ANTH_{RG} based on two signals, i.e. the ratio between far-red chlorophyll fluorescence under both red and green excitation.

Several applications of the Multiplex (Mx) sensor directly in the vineyards, for the manual determination of Anth evolution in a large number of bunches, have been reported (Ben Ghazlen et al., 2010; Baluja, Diago, Goovaerts, & Tardaguila, 2012a; Tuccio et al., 2011). It has been used for assessing the spatial variability of grape colour and for correlating this to several agronomic parameters, such as vine vigour and yield (Baluja, Diago, Goovaerts, & Tardaguila, 2012b).

The use of Mx as an on-the-go sensor mounted on a harvester has also been demonstrated (Bramley et al., 2011). Besides determining Anth, the Mx acts as a tool for characterizing the spatial variability of the vegetative status of the vineyard (Diago, Rey-Carames, Le Moigne, Fadaili, Tardaguila, & Cerovic 2016).

The Mx sensor can also represent a useful device for the winery laboratories as an alternative to UV–Vis spectroscopy colourimetric analyses of grape berries. In fact, there is a growing interest in adopting non-destructive techniques for the evaluation of grape quality, such as NIR hyperspectral imaging coupled with chemometrics (Chen, Zhang, Ning, Liu, Zhang & Yang, 2015; Nogales-Bueno, Baca-Bocanegra, Rodríguez-Pulido, Heredia & Hernández-Hierro, 2015; Zhang et al., 2017).

In general, any sensor aimed at being correctly quantitative must be calibrated against chemical determinations of the target molecules.

Previous studies have reported several calibrations of the Mx for Anth estimation in grape berries (Ben Ghazlen et al., 2010; Bramley et al., 2011; Tuccio et al., 2011; Baluja et al., 2012a; Agati et al., 2013; Ferrandino, Pagliarani, Carlomagno, Novello; Schubert & Agati 2017). The Anth levels investigated ranged from 0.7 – 0.8 mg g⁻¹ in Pinot Noir, Pinot Menier, Aleatico and Nebbiolo to about 2 mg g⁻¹ in Tempranillo, Merlot and Barbera, and up to 2.5 mg g⁻¹ in Shiraz and Cabernet Sauvignon. These predictiong prediction models were obtained from single- year

determinations and differed as a function of the cultivar. Ferrandino et al. (2017) also reported on the dependence of ANTH_{RG} on the cultivar Anth profile.

The morphological differences among grape varieties in size, weight and berry-skin thickness may have a dissimilar influence on the optical signals acquired by the Mx. Significant differences may also occur for the same variety from one winery to another, as they are influenced both by the terroir and by the different cultural practices. Variability among seasons in the meteorological conditions, particularly temperature and dryness, can induce water stress status in the vines, thus affecting berry size and Anth synthesis (Ojeda, Andary, Kraeva, Carbonneau, & Deloire, 2002; Deluc et al., 2009; Tuccio et al., 2011).

For this reason, an evaluation of both the cultivar and of the environmental and growing-place effects on the Mx calibration is required. In this paper, the berry anthocyanin concentrations have been correlated with the Mx indices (ANTH_R and ANTH_{RG}) in order to build different calibration models for i) individual cultivars, ii) the same cultivar in two different regions, iii) three cultivars on one site and, lastly, iv) all cultivars on two sites. The seasonal robustness of the models was evaluated by means of validation over three successive years. The Mx calibration models defined were then used to estimate Anth non-destructively from in-vineyard Mx measurements. Examples of applications concerning the comparison of Anth among different plots and years of the same cultivar per site, the comparison of Anth for the same cultivar between different sites and years, and the temporal evolution of Anth have also been reported.

2. Material and methods

2.1 Plant material and growing sites

The experiment was conducted over three consecutive years (2010-2012) at the Casale del Giglio (Aprilia, LT, Italy; 41°30'44.0"N, 12°44'44.2"E) and Castello Banfi (Montalcino, SI, Italy; 42.980339, 11.398776) wine estates. Casale del Giglio (CdG) is located in the Agro-Pontino Valley, 10 km from the Tyrrhenian Sea coast on a flat area (0-20 m of elevation) that is characterized by a maritime climate. Three cultivars from among the 160 ha of vineyards cultivated at CdG - namely, Merlot (ME), Petit Verdot (PV) and Syrah (SH) - were considered. The three varieties were bred using the Guyot or spur-pruned cordon system and the vines spaced 2.2 m × 0.8 m (inter- and intra- row). Two to three plots, which differed as regards clones and soil texture, were selected for each variety: 3A and 20 for Merlot, 30, 7 and 1B for Petit Verdot, and 2B, 6 and 21B for Syrah.

At the Castello Banfi (CB) estate, located in a territory that occupies 2,830 ha, 850 ha of which are for specialized vineyards, three red varieties and two to three plots per cultivar were considered. In particular: plots 23.04, 37.05 and 7.04 for cv. Merlot (ME), plots 12.07 and 10.13 for cv. Syrah (SH), and plots 24.09 and 7.03 for cv. Cabernet Sauvignon (CS). ~~In particular: plots 23.04, 37.05 and 7.04 for cv. Merlot (ME), plots 12.07 and 10.13 for cv. Syrah (SH), and plots 24.09 and 7.03 for cv. Cabernet Sauvignon (CS).~~ The three varieties were bred using the low spur-pruned cordon system and the vines were spaced 3.0 m × 0.8 m (inter- and intra- row). CB is located on an inner hillside (200-600 m a.s.l.) in southern Tuscany. The entire area is characterized by a temperate climate, with high exposure to the sun and to breezes and considerable variations between daytime and night-time temperatures.

The climatic conditions, global radiation, air temperature and rainfall recorded in the two regions during the 2010-2012 seasons are reported as online supplementary materials (Figure S1 and Table S1). The heat accumulation index for *Vitis vinifera* L., that is, the growing degree-days (GDD) index, was calculated according to Winkler (1974).

2.2 Optical sensor and indices

The Multiplex[®] (Mx) fluorimetric sensor (FORCE-A, Orsay, France) is described in detail elsewhere (Ben Ghazlen et al., 2010). It measures fluorescence emitted by chlorophyll in the 670-690 nm red (RF) and 720-780 nm far-red (FRF) spectral regions, under excitation with different light-emitting diode (LED) sources in the UV (375 nm) and visible (blue at 450 nm, green at 515 nm and red at 630 nm). Since the LED sources are pulsed and synchronized with the detection, the sensor is insensitive to ambient light and can be used directly in the vineyard. The wide detection area of the sensor (8-cm diameter) makes it possible for a signal to be acquired from a large area in each bunch. The acquisition time for a single bunch sample is less than 1 s. The collected data, which are visible on a real-time display, are stored on a secure digital card for further analyses.

The fluorescence indices used in this work are defined as:

$$\text{ANTH}_{\text{RG}} = \log (\text{FRF}_{\text{R}}/\text{FRF}_{\text{G}})$$

$$\text{ANTH}_{\text{R}} = \log(1/\text{FRF}_{\text{R}})$$

where FRF_{R} and FRF_{G} are the far-red chlorophyll fluorescence signals excited by red and green light, respectively (Ben Ghazlen, 2010). Signals were corrected for residual electronic offsets and normalized to a fluorescence standard (blue plastic foil, FORCE-A, Orsay France).

The selected bunches were extracted and analysed in the laboratory by means of spectrophotometric analysis, in order to evaluate the anthocyanin content. These data were then used to calibrate and validate the Mx optical sensor, as described here as follows.

2.3 Sampling for calibration and in field measurements

Samples of grapes were collected at different stages of ripening, from green to véraison until full ripeness, in order to cover as broad as possible a range of Anths content.

At CdG, the sampling dates were 20 and 26 August, 2, 9, 16 and 23 September for the 2010 campaign, 24 and 31 August, 8 and 14 September for the 2011 campaign, and 23 and 30 August, 12 September for the 2012 campaign. At CB, the sampling dates were 17 and 31 August, 10, 20 and 27 September, 5 and 12 October for the 2010 campaign; 29 August, 6, 12 and 21 September for the 2011 campaign; and 29 August and 11 September for the 2012 campaign.

The samples collected (2-3 bunches) were transported under cool conditions to the laboratory for further analysis.

On the same dates of sampling, in-field Mx measurements on bunches attached to the vines were performed as follows. At the beginning of the 2010 campaign, 2 to 4 rows per plot, depending on the plot size, were selected and marked. Twenty-five bunches per row were measured by the Mx, and the very same portion of each row was monitored over time until the harvest. The same rows of the same plots were also measured during the 2011 season.

2.4 Calibration of fluorescence indices against wet chemistry

From each single cultivar sample collected in the field, 19 berries (i.e. those filling the 5×10^3 mm² circular area of the sensor window) were selected and placed on a special support to simulate their position in the grape bunch, oriented with the apical side upwards (see Fig. S2d, supplementary information in Ben Ghazlen et al., 2010). They were then measured with the sensor at a fixed distance.

After being measured, each 19 berry-sample, the sample was weighed, homogenized in a blender for 20 sec and, then, extracted and analysed as follows. Each fresh sample was homogenized in a blender for 20 sec. In particular, Aa quantity of 5 grams of the sample

homogenate was extracted 1 h at room temperature by 50 mL of hydroalcoholic solution (70% ethanol and 30% Milli Q water acidified at pH 1.4 by HCl). The extract was and measured by means of the spectrophotometer Cary 50 (Agilent Technologies, Santa Clara, CA) that was provided with optical fibrespectrophotometry, after being filtered and diluted. The sample absorbance was measured at 5xx nm. Total Anths were expressed as malvidin-3-O-glucoside (oenin), using a calibration curve of five levels of concentration ($R^2 > 0.998$, with extinction coefficient ...). The spectrophotometric analysis of the diluted extracts was performed using a spectrophotometer Cary 50 (Agilent Technologies, Santa Clara, CA) that was provided with optical fibre. The resulting data were used to calibrate and validate both $ANTH_R$ and $ANTH_{RG}$ indices of the Mx optical sensor.

For each data set, calibration curves were built to fit the scatter plot between the anthocyanin concentrations determined by wet chemistry and the corresponding Mx fluorescence anthocyanin indices determined in the laboratory by means of the Mx. By following this procedure from the data collected during the 2010-2012 campaigns, different calibration curves (as previously described) were calculated and compared. The data were processed using the SigmaPlot 12.5 programme (SYSTAT software, Inc. SigmaPlot for Windows).

2.5 Calibration for in field application

As observed previously (Ben Ghazlen et al., 2010; Bramley et al., 2011), the Anth Mx indices measured on a bunch of grapes were shifted as compared with those measured on berries from the same bunch detached and arranged in a cluster-like holder, as described above. The reason for this is not yet known, but it is most likely due to a geometrical effect that can change the amount of berry-scattered light collected by the sensor in the two different configurations. However, by comparing bunch measurements in the field with berry measurements of sampling

from the same sub-plot, a fine correlation between the two sets of Anth indices was found (data not shown [see supplemental material](#)). This correlation was considered in order to convert the calibration curves built with the optical measurements in the laboratory into those to be used in the field.

2.6 Solvents and chemicals

Water was purified with the use of a Milli-Q system (Bedford, MA, USA). The ethanol and hydrochloric acid used for the grape extractions were supplied by Sigma-Aldrich. The authentic standard of malvidin 3-O- β -D-glucoside or oenin (> 90% HPLC) was purchased from Extrasynthese (Nord Genay, Lyon, France).

2.7 Statistical analysis

Statistical analysis was carried out using the SigmaPlot Program 12.5 (Systat Software, Inc. SigmaPlot for Windows), and statistical differences were evaluated by means of t-test analysis or by the all-pairwise, multiple-comparison Tukey ANOVA test. P values of <0.05 were considered statistically significant. The accuracy of the calibration curves was described by the Root Mean Square Error of Calibration (RMSEC) and the coefficient of determination (R^2). The Root Mean Square Error of Prediction (RMSEP) was calculated in order to evaluate the predictive ability of the regression models. The Relative Error (RE) was defined as the percentage of RMSEP relative to the mean Anth values measured at harvest (calculated over the last 2 weeks before harvest), in order to compare the model performances with each other. A cross-validation procedure was used to evaluate the accuracy of the calibration curves. For each data set, two-thirds of the whole values were classified in categories of 3-4 data with increasing Anth values. From each category, one value was randomly chosen to define the

validation data set corresponding to one-third of the entire data. The remaining two-thirds were used as a calibration data set. The procedure was repeated five times; the average values ($\pm$ SD) of R^2 and RMSEC for the models of calibration and the RMSEP and RE for the model validation were then calculated.

3. Results and discussion

3.1 Calibration models

In order to define a robust calibration of the Mx sensor for evaluating the Anth content of the grape berries, a heterogeneous set of data obtained from various cultivars and from two different geographical regions was employed.

Here as follows, the Anth concentration was expressed per berry mass (Anth_g), i.e. milligrams of Anth per grams of berry fresh weight, since it is the main unit used in winery laboratories. However, some considerations on Anth_m , the Anth concentration per berry skin mass, for an interpretation of seasonal effects on the calibration models are also presented. In particular, Anth_g was the parameter directly measured by means of berry extraction and spectrophotometric determination, while Anth_m was calculated by using the berry mass and the CUBA (Converter of Units of Berry Anths) software (Cerovic, Latouche, Nguyen, Fadaili, Le Moigne, & Ben Ghazlen, 2014).

~~3.1 Calibration models~~

3.1.1 Individual cultivars per site

Figure 1 shows the relationship between the Mx indices and the total anthocyanin content per berry mass (Anth_g) of sampling collected for all the cvs and the two investigated sites during the entire 2010 season. All points of each data set were registered during and after véraison.

As expected on the basis of previous studies, the ANTH_R was positively correlated to Anth_g over the entire range of data and for all cultivars. The calibration curve derived for ANTH_R was represented in any case by a homographic function, with $R^2 > 0.9$ (see data reported in Table S2 of Supplementary materials).

ANTH_{RG} increased up to a maximum with an increase in the Anth_g during véraison, and then showed an opposite trend from complete véraison to harvest time. This bi-phasic behaviour is

in accordance with the theory and definition of the index, as previously reported (Ben Ghazlen

et al., 2010). The analytical function representing the ANTH_{RG} versus the Anth_g relationship

was the difference between two exponential decaying functions, as depicted in Fig. 1 with the

use of dotted lines. Calibration of the ANTH_{RG} can be problematic, since the bi-exponential

function cannot be inverted analytically and even a numerical solution to it can cause ambiguity

around véraison or at lower Anth_g values. It was therefore suggested to use the ANTH_{RG} index

only when véraison had been completed (Bramley et al., 2011). For all cvs, the calibration

curves derived for ANTH_{RG} , when considering only post-véraison data (closed triangles in Fig.

1), were represented by an exponential decaying function. In all cases except cv. CS ($R^2 =$

0.543), at least 80% of the Anth_g versus ANTH_{RG} distribution was explained by the exponential

function (see data reported in Table S2 of Supplementary materials). Significant differences

appeared in the ME calibration curves for both indices between the two sites (Figs. 1C and D

and Table S2), while SH calibration curves at CdG and CB were rather similar (Figs. 1A and B

and Table S2).

The lowest RMSEC (0.075 mg g^{-1}) was found for ANTH_R in ME at CB, while the highest (0.339 ~~0.229~~ mg g^{-1}) was obtained for ANTH_{RG} in ~~SH at CB~~ **PV at CdG**. A better comparison of the model performances can be obtained by considering the RMSEP and RE values from the cross-validation process reported in Tables 1 and 2, as averages of five repetitions. For both Mx indices, the calibration curves built with 2/3 of the data were comparable to those built with the entire set of data in terms of R^2 and RMSEC (compare values in Tables 1 and 2 with those in Table S2). By using ANTH_R, the best model for Anth_g prediction was obtained for SH at CB (RE = 7.4%) and the highest RE was recorded for SH at CdG (13.4%) (Table 1). The ANTH_{RG} model produced higher RE than the ANTH_R one, ranging from 8.7% (ME at CB) to ~~49~~ **16**% (SH at CB).

3.1.2 Inter-site for individual cultivars

For the two cvs present on both the CdG and CB estates, i.e. Shiraz and Merlot, an inter-site calibration model was considered, using the single cv data from the two sites. The calibration curves obtained were validated on the same data set considered for the validation of the previous single-cv single-site models. The results of this analysis are reported in Tables 1 and 2, for ANTH_R and ANTH_{RG}, respectively. The inter-site ANTH_R calibration model was very similar to that of the individual cvs (compare R^2 and RMSEC between the first two rows for SH and ME in Table 1).

The percentage relative error was less than 13.5%, close to and sometimes even better than the values obtained from the single-site calibration models.

On the whole, no significant differences were observed (P value >0.05) between the inter-site models and the single-cv models.

When considering the inter-site ANTH_{RG} models, it was found that the calibration parameters were ~~tendentially inferior as compared with~~ similar to those of the single-site ones (Table 2). ~~However, the differences~~ between the two models ~~were~~ as significant ($P < 0.001$) only for ME at CB, with R^2 reduced from 0.92 to 0.78 and RMSEC, RMSEP and RE almost doubled. In all the other cases, SH at CdG and CB and ME at CdG, no significant differences were observed.

3.1.3 Inter-cultivar per site

Here, inter-cultivar calibration models for each of the two regions, i.e. SH, ME and PV at CdG and SH, ME and CS at CB, were considered. For ANTH_R models, again no statistically significant ($P > 0.05$) variations were found in the calibration and validation parameters as compared with the individual cv models (Table 1). The trend was slightly worse for SH and ME at both sites, but was better for PV and CS. These data suggested that for the three cvs investigated, a sufficient accuracy in the Anth_g prediction could be obtained in the same geographical area by using a single inter-cv model.

The same conclusion could be drawn for the ANTH_{RG} models, with the sole exception of ME at CB, for which the RE of ~~13.2~~ 14.1% given by the inter-cv calibration was significantly ($P =$ ~~0.008~~ 0.006) higher than that from the individual-cv model (8.7%) (see Table 2).

3.1.4 Global (inter-cultivar and inter-site) calibration models

Lastly, an evaluation was performed of global calibration models that included all the cvs at both sites. The comparison of these models with the individual-cv models led to the same conclusion as before. The accuracy of prediction was essentially the same for the two models, with the exception of the ANTH_{RG} relative to SH and ME at CB, the global model for which

produced a RE of 19% and 17.417.3%, respectively, a significantly ($P < 0.001$) different from the values from the 8.7% recorded for the individual-cv model (Tables 1 and 2).

~~From this analysis, it resulted that~~ Therefore, a general calibration model can be used for the prediction of Anth_g in grape berries, with relative errors no higher than 14.1% and 21.319% for ANTH_R and ANTH_{RG}, respectively, depending on both the cv and the growing site.

Figures 2A and 2B show the scatter plots of the Anth_g quantitative data and the ANTH_R and ANTH_{RG} indices, respectively, which were acquired during the 2010 campaign. The global calibration curve derived for ANTH_R was still well represented ($R^2 = 0.932$) by a homographic function (Fig. 2A) with equation:

$$\text{Anth}_g = \frac{\text{ANTH}_R}{(1.5571 - 0.2832 \text{ ANTH}_R)} \quad (1)$$

For the ANTH_{RG} index, the data points were more dispersed (Fig. 2B) but still fairly ($R^2 =$ 0.8040.797) well fitted by a decaying exponential function, with equation:

$$\text{Anth}_g = \text{2.22542.1924} e^{-2.48155672 \text{ ANTH}_{RG}} \quad (2)$$

3.1.5 Seasonal effects

Seasonal robustness of the 2010 ~~calibration curves for predicting berry Anth~~ models was evaluated by plotting ~~them~~ the calibration curves together with the data sets acquired in 2011 and 2012, as reported in Figs. 2C and 2D for ANTH_R and ANTH_{RG}, respectively.

In considering ANTH_R, a significant shift in the data points, from véraison on, occurred among the seasons (Fig. 2C). As compared with 2010, the shift was greater for 2011 than for 2012. In considering ANTH_{RG}, the data sets of the three seasons were in agreement, even if the points for 2012 were rather dispersed, as can be seen in Figure 2D.

371 Validation of the 2010 calibration curve on 2011 and 2012 data sets produced RMSEP for
372 ANTH_R of 0.4 and 0.31 mg g⁻¹, respectively, which corresponded to relative errors of 33% and
373 26%. For ANTH_{RG}, RMSEP was 0.21 and 0.26 mg g⁻¹, with relative errors of 48.17% and 22%,
374 during the 2011 and 2012 seasons, respectively.

375 A conclusive explanation for this seasonal variability in the relationship between Anth_g and the
376 Mx Anth indices cannot be provided at present. One possible reason may “simply” be related
377 to a way of expressing the Anth content in the berries that can affect its correlation with the
378 non-destructive optical measurements. Since Anths are localized into the berry skin, using the
379 skin mass based (Anth_m) or the berry mass based (Anth_g) concentrations can make a significant
380 difference. To verify this aspect, the 2010 calibration curves were calculated again for the Mx
381 indices by using Anth_m and were newly analysed, in comparison with 2011 and 2012 data sets.
382 The conversion of Anth_g to Anth_m required a knowledge of the Relative Skin Mass (RSM),
383 which was not available for our set of data. However, a simulation could be performed by means
384 of the internet-available CUBA software tool (Cerovic et al., 2014) and by considering the RSM
385 values for specific cultivars found into the literature. Within the Anth concentration conversion
386 process, the previously reported changes in RSM during the growing season, from véraison to
387 harvest, must be considered. In fact, according to Ojeda et al. (2002), the Shiraz RSM was
388 calculated to vary from about 6% at pre-véraison to 9% at harvest, yet Keller & Hrazdina (1998)
389 reported a change in RSM for Cabernet Sauvignon of between 8% and 10% within 6 weeks
390 after véraison.

391 In Figs. 2E and 2F, the results of the simulation for the cv Shiraz, that considered data points
392 from both the CdG and CB estates, are reported. For the Anth_g to Anth_m conversion, RSM
393 values of 7% during véraison and 9% after véraison were applied.

The new 2010 calibration for both $ANTH_R$ and $ANTH_{RG}$ against $Anth_m$ in SH was similar to that against $Anth_g$, with homographic ($R^2 = 0.92$) and decaying exponential ($R^2 = 0.76$) fitting curves, respectively (to be compared with values in the second row, calibration column of Tables 1 and 2). Figure 2E shows that the shift among data points of the three seasons concerning $ANTH_R$ remained, while the 2010 calibration curve for $ANTH_{RG}$ was well overlapped to the corresponding data points for 2011 and 2012 (Fig. 2F). Therefore, the seasonal effect on the calibration curves was substantially independent of the $Anth$ concentration units.

Since RSM is significantly affected by the water stress and by the growing season, a simulation was performed of which change in RSM for Shiraz during the years of 2011 and 2012 could have had the best agreement with the 2010 calibration curve. Hence, for each index and year, the RMSEP was calculated as a function of the RSM, as reported in Fig. 3.

The minimum RMSEP for $ANTH_R$ (Fig. 3A) was registered for RSMs of 6.8% and 7.8% in 2011 and 2012, respectively. These values were lower than the 9% RSM assumed after véraison in 2010, an occurrence that is explainable by less favourable growing conditions for vines in 2010 ~~and~~ relative to 2011 and 2012. In fact, it was proved that when vine growth is reduced, there is an increase in the RSM of the berries (Ojeda et al. 2002; Roby, Harbertson, Adams, & Matthews 2004; Cooley, Clingeleffer & Walker 2017).

Under water deficit, RSM of Shiraz berries can reach values of more than 10% (Ojeda et al., 2002). Roby et al. (2004) showed that, under low irrigation regimes, Cabernet Sauvignon berries had a RSM of 18% as compared with the 13% of the controls.

There is also a high inter-season variability of RSM (Palliotti, Gatti, & Poni, 2011). Bucchetti, Matthews, Falginella, Peterlunger, & Castellarin (2011) reported a variability in RSM from 9.6% to 12.1% at harvest in the cv Merlot between two consecutive seasons. Marked changes

in RSM can also be induced by vine management practices that are used to control vine vigour, such as defoliation (Poni, Bernizzoni, Civardi, & Libelli, 2009; Palliotti et al., 2011).

For ANTH_{RG} (Fig. 3B), the minimum RMSEP was obtained at similar RSM, 9% and 8.8% in 2011 and 2012, respectively, with no change compared with the 2010 values, which were considered from véraison to harvest. This result was in good agreement with the overlapping between the 2010 ANTH_{RG} calibration curve and the data from the two following seasons shown in Fig. 2F.

To explain the seasonal effect on the calibration curves, we hypothesize that the different meteorological conditions for each year (see Fig. S1 and Table S1, Supplementary material) affect the berry RSM and this induced change has an effect on the ANTH_R versus Anth_m relationship.

Indeed, the growing degree days (GDD), representing the heat accumulation during the April-October period for 2010, was markedly lower than in 2011 and 2012 (1935 versus 2238 and 2316 at CB; 2247 versus 2350 and 2395 at CdG), and supported relatively higher RSM values in 2010 than in later years.

Finally, the ANTH_{RG} index is less sensitive than ANTH_R to the change in RSM, at least for the SH cultivar.

3.1.6 Closing remarks

The global model built with 2010 data from all cultivars at both sites can represent a valuable choice, thanks to the acceptable precision of prediction with maximal relative errors of about 14% and 21.19% for ANTH_R and ANTH_{RG}, respectively. This precision is very close to that obtainable from the single-cultivar models (with RE $\leq$ 13.4% and $\leq$ 19.16% for ANTH_R

and ANTH_{RG}, respectively) and in some cases is even better, which suggests the possibility of applying the global model to an even wider range of cultivars and sites.

ANTH_R calibration curves, both global and single-cultivar, were not stable with the years, with RE values of over 30%, apart from the SH case in 2012 (16.3%).

The constancy of the ANTH_{RG} calibration curves over the years was satisfactory, with RE remaining around 20% in 2011-2012 for the global model. If we consider the single cultivar models, in 2011 and 2012 RE was 9.8% and 9.37%, respectively, for SH and 13.4% and 26.6%, respectively, for ME.

Our analysis indicates that the Mx sensor represents a valid green analytical tool to be used in winery laboratories as a complementary method to the time-consuming, costly, and environmentally-unfriendly standard Anth analyses.

3.2 In field applications

Here as follows are outlined some examples of applications of the calibrated Mx sensor for Anth quantitative estimates in the vineyard, such as i) a comparison of Anth_g among different plots and years of the same cultivar per site; ii) a comparison of Anth_g for the same cultivar between different sites and years; iii) a temporal evolution in Anth_g.

We used the ANTH_{RG} prediction models for the in-field application of the sensor, because these are less susceptible to seasonal effects as compared with ANTH_R ones (see Figs. 2C-F). The in-field calibration curves for ANTH_{RG} were derived from the calibration curves built in the lab (see paragraph 2.5), by considering the overall data collected within the 3 seasons (2010, 2011 and 2012). The only exception was that of CS (2010 and 2011 data). All models were described by decaying exponential curves, with equations and parameters detailed in the following:

Global calibration curve (all cvs, both sites):

$$\text{Anth}_g = 3.01842.9518 e^{-5.05884.9198 \text{ANTH}_{\text{RG}}} \quad (R^2=0.794787; \text{RMSEC}=0.220215 \text{ mg g}^{-1}) \quad (3)$$

SH (single cv, both sites):

$$\text{Anth}_g = 3.92453.5062 e^{-5.84685.2542 \text{ANTH}_{\text{RG}}} \quad (R^2=0.8602; \text{RMSEC}=0.184162 \text{ mg g}^{-1}) \quad (4)$$

ME (single cv, both sites):

$$\text{Anth}_g = 2.6988 e^{-4.6415 \text{ANTH}_{\text{RG}}} \quad (R^2=0.726; \text{RMSEC}=0.207 \text{ mg g}^{-1}) \quad (5)$$

PV (single cv, CdG):

$$\text{Anth}_g = 3.1751 e^{-5.9804 \text{ANTH}_{\text{RG}}} \quad (R^2=0.870; \text{RMSEC}=0.219 \text{ mg g}^{-1}) \quad (6)$$

CS (single cv, CB):

$$\text{Anth}_g = 2.6127 e^{-3.8054 \text{ANTH}_{\text{RG}}} \quad (R^2=0.614; \text{RMSEC}=0.211 \text{ mg g}^{-1}) \quad (7)$$

These curves were applied in order to estimate Anth_g from Mx data collected during the 2010 and 2011 campaigns. Values obtained from the global curve were compared with those from the single cv curves, and a difference in Anth_g no greater than 18% was found.

It was below 10% in about 70% of cases out of a total of 32 plots analysed. Greater differences were observed only for SH of the CB winery (both years), SH of the CdG winery in 2010, and one plot of CS in 2011.

The global curve underestimated SH Anth_g with no significant differences for CdG in 2011, but with significant differences in all the other cases ($P \leq 0.026$).

ME Anth_g was overestimated by the global curve, but at no more than 5%; moreover, only 3 cases out of 10 reported significant differences (data not shown).

Therefore, and as shown for the laboratory conditions, the choice of the global calibration curve appears to be adequate. However, the single-cultivar calibration can be adopted in the case of higher accuracy requirements for the Anth determination.

3.2.1 ~~Comparison of~~ Anth content among different plots and years of the same cultivar per site

The Anth_g content evaluated on different plots of the cultivars monitored over two consecutive campaigns (2010 and 2011) at the CdG and CB estates is reported in Figs. 4A-F. The number of bunches measured by the Mx sensor for each plot varied between 85 and 340, depending on the size of the plot. The Anth_g content was calculated by using a single cultivar calibration curve and averaging Mx measurements taken at the moment of two temporal acquisitions, namely at harvest and one week previously. On the whole, as depicted in Fig. 4, one of the main advantages of using the sensor is to show differences among samples, even though there is an extensive variability. In general, more extensive sampling corresponds to a greater opportunity to see differences, even when these are very small.

ME at CdG was the most homogeneous cultivar among the plots considered (Fig. 4B).

Anth_g of SH at CdG was constant among the plots in 2011, but not in 2010 (Fig. 4A). The opposite was observed for PV (Fig. 4C). For each of the three cvs investigated at CB, significant differences were always observed among the plots (Figs. 4D-F).

Variability in Anth_g among plots of the same cv on a single growing site is a common feature. This may depend on the clones/rootstocks, the soil characteristics (Yokotsuka, Nagao, Nakazawa, & Sato, 1999), water (Deluc et al., 2009) and nutrient (Keller, & Hrazdina, 1998) availability, and then on the vegetative status of the vines. An inverse relationship usually exists

between grape Anth content and vine vigour (Cortell, Halbleib, Gallagher, Righetti, & Kennedy, 2007), this last showing a high spatial variability even within the same plot (Baluja et al. 2012b).

A significant difference between the two seasons was always registered with higher Anth_g in 2010, except for plot 7 of PV at the CdG winery, even if at the limit of significance (P=0.061). This result confirmed the less favourable growing conditions experienced by the vines in 2010 compared to 2011, as suggested by the comparison of GDD between the years (see Table S1 Supplementary material).

Extensive variations in grape Anth among the seasons have often been observed in other studies as being linked to the meteorological conditions (mainly to precipitation and to temperature) (Spayd, Tarara, Mee, & Ferguson, 2002; Guidoni, Ferrandino, & Novello, 2008; Baluja, Tardaguila, Ayestaran, & Diago, 2013).

3.2.2 ~~Comparison of~~ Anth content for the same cultivar between different sites and years

The estimated Anth_g averaged over several plots for the same cultivar (SH or ME) at each location in the proximity of the harvest in 2010 and 2011 are compared in Fig. 4G. In this case, the number of bunches sampled by the Mx for each determination increased, and ranged from 216 (ME at CdG in 2011) to 717 (ME at CB in 2011).

SH was the most heterogeneous cultivar, being characterised by significant differences between seasons and sites. The Anth_g was higher at CB than at CdG for both years. The average value of Anth_g content in ME plots differed significantly between the wineries in 2010, but not during the 2011 season. Again, this fluctuation can be ascribed to the combination of differences on the two sites in clone characteristics, soil texture, climatic conditions and cultural practices

(Guidoni et al., 2008; Poni et al., 2009). Generally speaking, in 2010 the Anth_g accumulation was larger than it was during the 2011 season.

3.2.3 Temporal evolution of Anth content

The temporal evolution of Anth content in the cv PV (plot 7) during the 2010 and 2011 campaigns is reported in Fig. 4H, where each point represents the average of 20-45 bunches. In 2010, the accumulation of Anth was delayed as compared with 2011, and increased significantly from DOY 232 to harvest (DOY 259). In 2011, Anth_g had already reached a plateau (1.9-2.0 mg g⁻¹) at the beginning of the monitoring (DOY 236), and remained constant for 2 weeks. After that, a tendency to decrease appeared even if it was not statistically significant. The 2010 Anth_g exceeded the 2011 value at harvest time. The kinetics recorded clearly showed the seasonal climatic differential effect on the synthesis of Anth.

4. Conclusions

An accurate determination of the Anth content in grape berries is fundamental for viticulturists and oenologists. Since the grape Anth content has a large biological variability, the introduction of optical sensors for its non-destructive evaluation is extremely advantageous: it makes it possible to have a large number of samplings, representative of the entire crop, directly measured in the vineyard within relative short times. The Mx fluorescence sensor is becoming widely used as a tool in viticulture, and for this a complete characterization of its performances

is required. In this paper, the Mx has been calibrated against chemical determinations of the target molecules, using different models.

The completeness of the analytical approach presented herein is supported by the use of data from four cultivars, two geographical areas, and three consecutive years of production, in order to guarantee the highest repeatability and reproducibility.

The main results concern: 1) the definition of a very general global calibration curve essentially similar, in Anth prediction, to single-cultivar curves and rather independent of grape cultivar and growing site; 2) proof of the seasonal robustness of the ANTH_{RG} calibration curves, as well as of the seasonal susceptibility of ANTH_R calibration curves due to climatic changes in the berry morphology (Relative Skin Mass); 3) the demonstration of the practical utility of a fluorescence sensor for precision viticulture purposes.

Thanks to the massive sampling performed in the vineyards by the optical sensor, it was possible to show differences in Anth among plots, despite an extensive biological variability.

Acquisition of these online data can be useful ~~when-for~~ making decisions on the best ~~period-for~~ ~~the~~ harvest time or for better defining the vine management ~~of the vines~~. Mapping large plots by means of the Mx proximal sensing is also possible in order to perform selective harvesting and to produce wines with a differentiated quality.

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582 **Conflict of interest:** none

583

Appendix

Supplementary material about Climate conditions: Figure S1 and Table S1

Captions to Figure and Table

Fig. S1 Meteorological conditions (global irradiance, air temperature, rainfall) registered by means of weather stations close to the investigated wineries, Casale del Giglio (CdG) and Castello Banfi (CB), during the ~~years~~ 2010-2012 seasons.

Table S1. Growing degree-days (base 10°C) (GDD), cumulative rainfall (mm), cumulative solar radiation and number of days with $T \geq 35$ °C at the two growing sites during the 2010-2012 seasons.

Details about the calibration models: Table S2

Caption to Table

Table S2. Calibration models for the $ANTH_R$ and $ANTH_{RG}$ indices against the destructively assessed ~~berry~~ $Anth_g$ (expressed as $mg\ g^{-1}$) berry content for each cultivar grown at Casale del Giglio (CdG) and Castello Banfi (CB) farms in 2010. SH=Shiraz; ME=Merlot; PV=Petit Verdot; CS= Cabernet Sauvignon.

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Captions for Figures

Fig. 1 Multiplex calibration curves for individual cultivars. The scatter plots of Anth_g versus ANTH_R are described by homographic functions $y = x/(a + bx)$ (see Table S2 of Supplementary material for fitting parameters); the scatter plots of Anth_g versus ANTH_{RG} post veraison are described by exponential functions $y = ae^{-bx}$ (see Table S2 of Supplementary material for fitting parameters). Cultivars grown at Casale del Giglio: SH (A), ME (C) and PV (E). Cultivars grown at Castello Banfi: SH (B), ME (D) and CS (F).

Fig. 2 Global (inter-cultivar and inter-site) anthocyanin calibration curves for the Mx ANTH_R (A) and ANTH_{RG} (B) indices ~~from recorded in the 2010 campaign~~. The homographic function in (A) has equation: $\text{Anth}_g = \frac{\text{ANTH}_R}{(1.5571 - 0.2832 \text{ ANTH}_R)}$ ($R^2 = 0.932$). The exponential function in (B) has equation: $\text{Anth}_g = 2.2254 e^{-2.5672 \text{ ANTH}_{RG}}$ ($R^2 = 0.802$). Comparison between the 2010 global curve and the 2011-2012 data sets ~~from the 2011 and 2012 campaigns~~ for ANTH_R (C) and ANTH_{RG} (D) indices with the anthocyanin concentration reported on a berry mass basis (Anth_g). Comparison between the 2010 individual cultivar calibration curve for Shiraz (both sites) and the 2011-2012 data sets ~~from the 2011 and 2012 campaigns~~ for ANTH_R (E) and ANTH_{RG} (F) indices with the anthocyanin concentration reported on a skin mass basis (Anth_m).

Fig. 3 Root Mean Squared Error of Prediction (RMSEP) for the 2010 SH calibration curves (reported in Fig. 2E-F) validated over the 2011 and 2012 data sets as function of the berry Relative Skin Mass (RSM) for ANTH_R (A) and ANTH_{RG} (B).

Fig. 4. Anth_g content evaluated on different plots of the cultivars monitored over two consecutive campaigns (2010 and 2011) at the Casale del Giglio (CdG) estate: SH (A), ME (B) and PV (C), and at the Castello Banfi (CB) estate: SH (D), ME (E) and CS (F). Bars accompanied by asterisks indicate a significant difference ($p < 0.001$) between years for the same plot; different small letters (for 2010 campaign) and different small letters with apex (for 2011 campaign) mean significantly different Anth_g ($p < 0.001$). (G) Averaged estimated Anth_g content in cultivars SH and ME from 2010 and 2011 campaigns at CdG and CB wineries. Bars accompanied by different small letters indicate significantly different Anth_g ($p < 0.001$). (H) Temporal evolution of Anth content in cv PV (~~Plot~~plot PV7) during the 2010 and 2011 seasons. Points with different capital letters indicate a significant difference ($p < 0.001$) between years for the similar collecting date; different small letters mean a significant difference ($p < 0.001$) among DOY (occurring only in 2010).

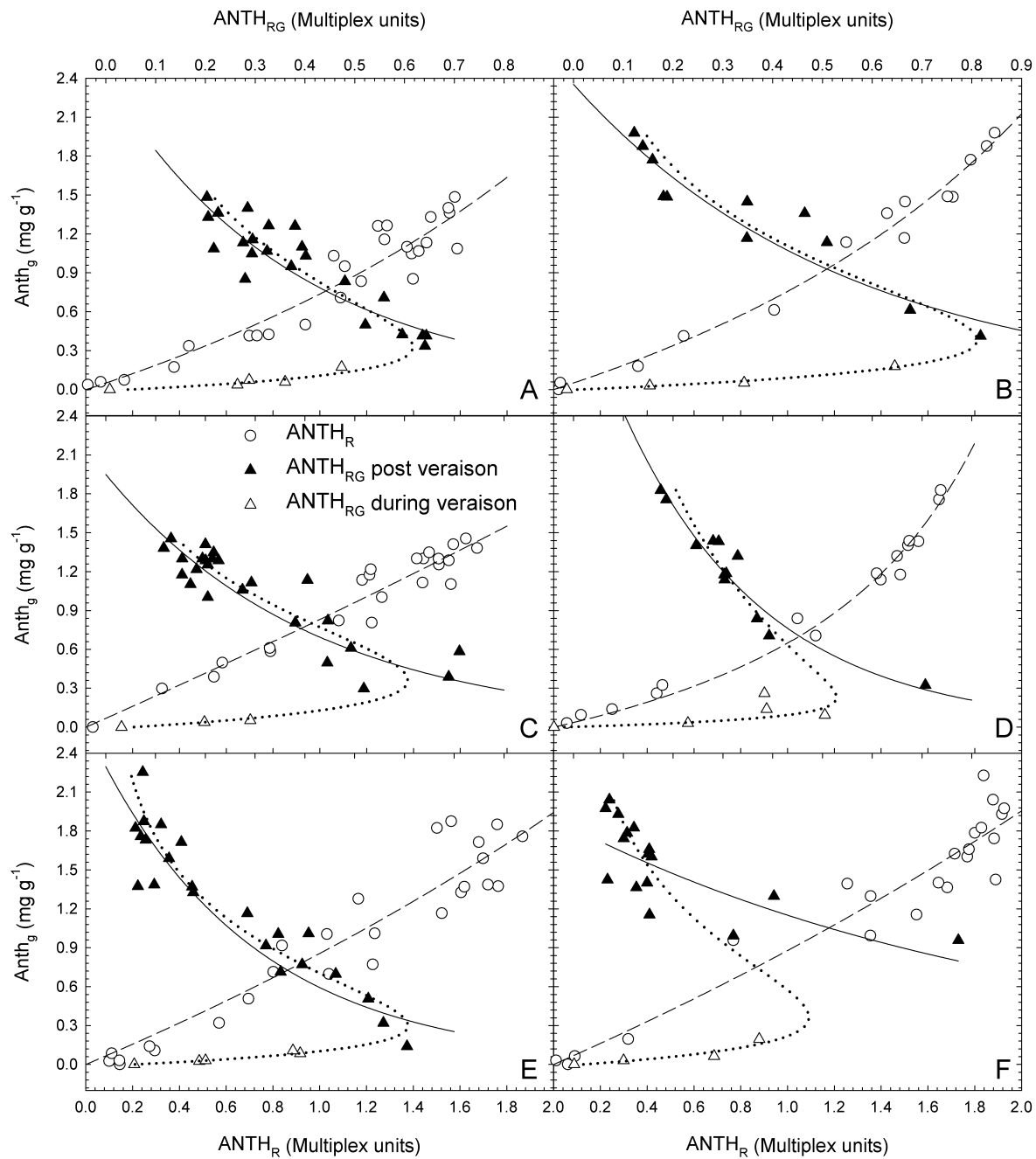


Figure 1

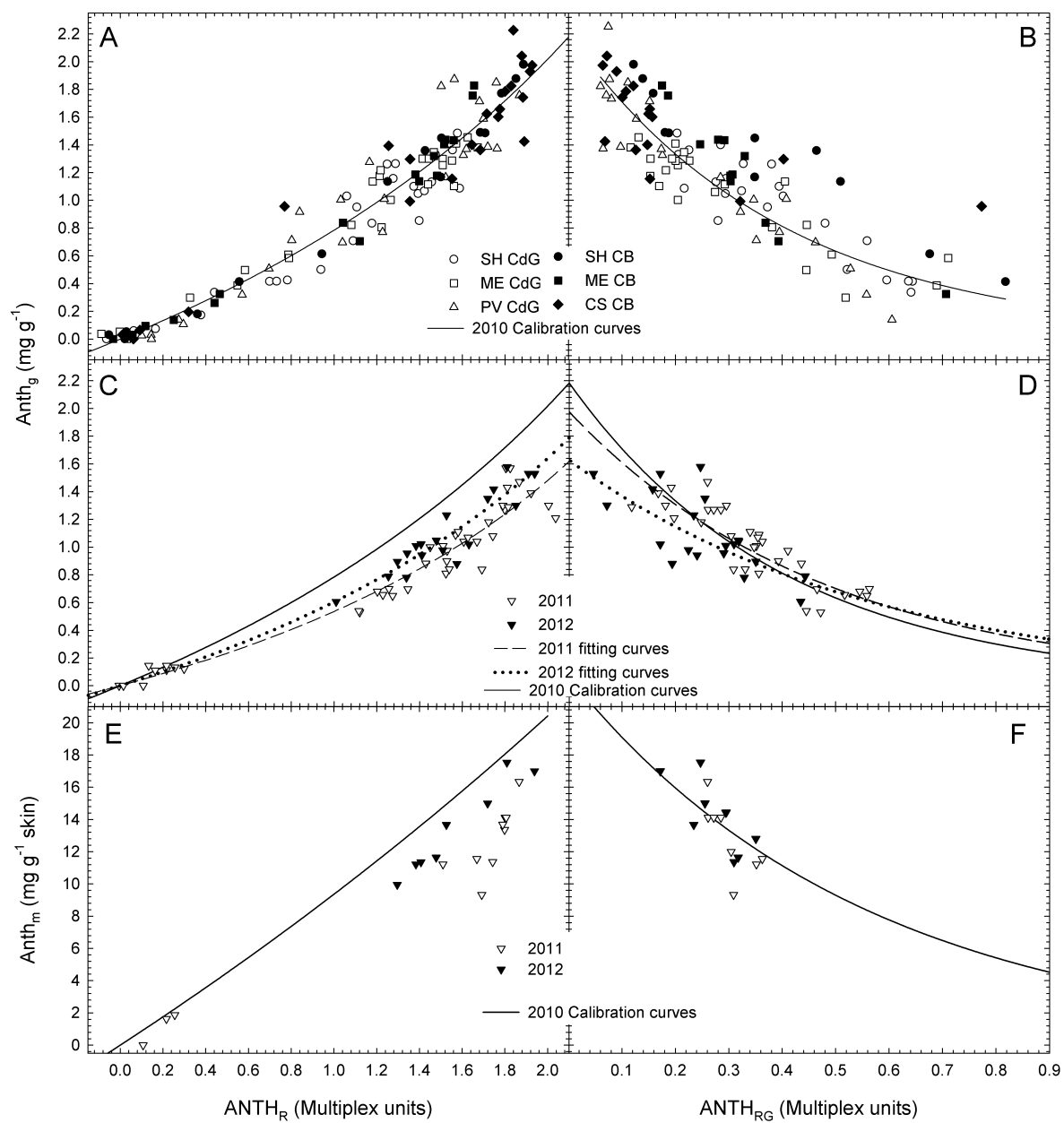


Figure 2

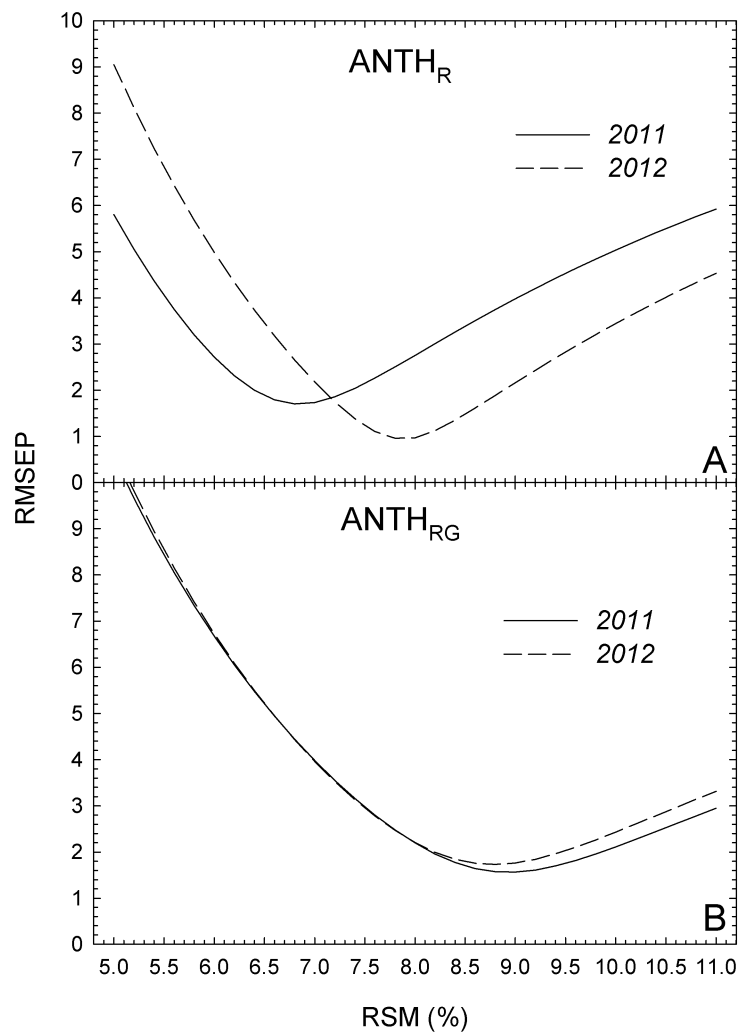


Figure 3

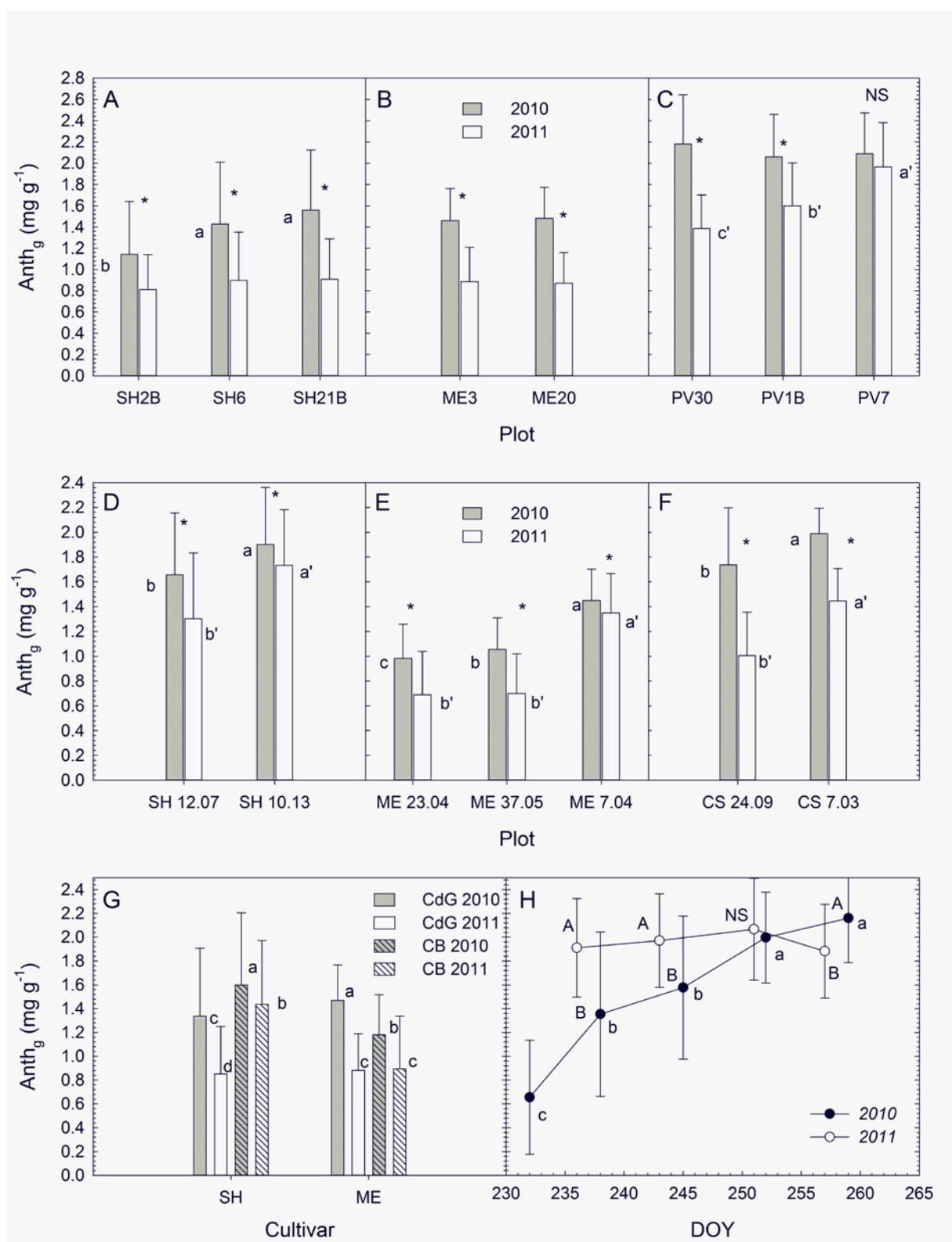


Figure 4